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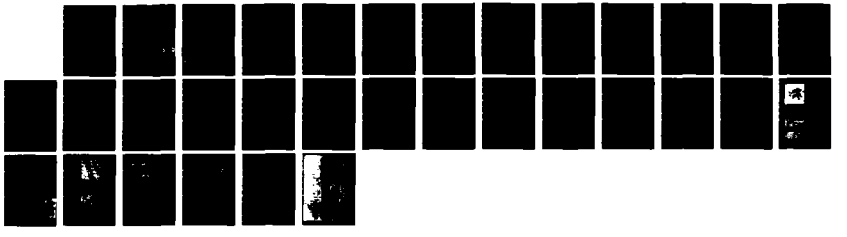

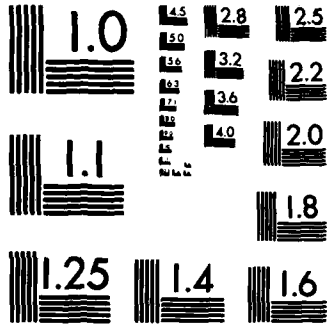

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Platelet-endothelial cell interactions are important for maintaining normal hemodynamics. The intact endothelial cell lining is considered non-thrombogenic but following disruption of the lining platelets will bind to the subendothelium. There is also much conjecture concerning the affinity of platelets for damaged endothelial cells. A model is described for the study of platelet-aorta vessel wall interactions following freeze-thaw insult. Using this model, control aortas (37°C) perfused with platelet rich plasma (PRP) or gel filtered platelets

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(GFP) showed no platelet-endothelial cell interactions, though some platelets did adhere to areas of exposed subendothelium. Following freeze-thaw insult (-15°C) or -20°C), the endothelial lining was grossly disrupted. The remaining endothelium was severely damaged, demonstrating holes and pits in the plasma membranes and separation of adjacent cell borders. Platelets readily adhered to the subendothelium, but were rarely noted in sole contact with the damaged endothelium. Platelet binding did not result in morphological changes, degranulation or aggregation. Using transmission electron microcopy, platelets were noted in contact with amorphous material and microfibrils but not collagen fibers of the subendothelium. It is concluded that this model is suitable for the in vitro study of certain hemodynamic phenomena associated with blood vessel freeze-thaw injury. In addition, freeze-thaw damage in this in vitro model, indicated that platelet-vessel wall interactions were limited to areas of exposed subendothelium.

Title: An In Vitro Model for the Study of Platelet-Vessel Wall Interactions Following a Freeze-Thaw Injury

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ABSTRACT:

Platelet-endothelial cell interactions are important for maintaining normal hemodynamics. The intact endothelial cell lining is considered non-thrombogenic, but following disruption of the lining platelets will bind to the subendothelium. There is also much conjecture concerning the affinity of platelets for damaged endothelial cells. A model is described for the study of platelet-aorta vessel wall interactions following freeze-thaw insult. Using this model, control aortas (37°C) perfused with platelet rich plasma (PRP) or gel filtered platelets (GFP) showed no platelet-endothelial cell interactions, though some platelets did adhere to areas of exposed subendothelium. Following freeze-thaw insult (-15°C or -20°C), the endothelial lining was grossly disrupted. The remaining endothelium was severely damaged, demonstrating holes and pits in the plasma membranes and separation of adjacent cell borders. Platelets readily adhered to the subendothelium, but were rarely noted in sole contact with the damaged endothelium. Platelet binding did not result in morphological changes, degranulation or aggregation. Using transmission electron microscopy, platelets were noted in contact with amorphous material and microfibrils but not collagen fibers of the subendothelium. It is concluded that this model is suitable for the in vitro study of certain hemodynamic phenomena associated with blood vessel freeze-thaw injury. In addition, freeze-thaw damage in this in vitro model, indicated that platelet-vessel wall interactions were limited to areas of exposed subendothelium.

KEY WORDS: Platelet, endothelial cell, freeze-thaw, subendothel um.

INTRODUCTION:

The intact endothelial cell lining of blood vessels is generally considered nonthrombogenic (13,21,26,28). Damage or removal of this exposes subendothelial components and initiates the coagulation mechanisms (7,21). Activation of platelets leading to their adhesion and aggregation is the first and perhaps most important step in the coagulation process. Although the thrombogenicity of subendothelial components such as collagen are well known (3,4,8,30,27,33). the ability of damaged endothelial cells to initiate platelet attachment and/or aggregation is subject of debate. Whereas, some investigators demonstrate platelet interaction with damaged endothelial cells (2,11,15,19), others are unable to confirm these observations and report that platelets do not interact with severely damaged endothelial cells 91,9,31,32,34).

In the case of frostbite, endothelial cells are often damaged or removed by a freeze-thaw injury to the blood vessels of an extremity. This injury initiates coagulation mechanisms which eventually lead to hemostasis following thaw (23). The extent of hemostasis is ultimately responsible for determining post-thaw necrosis and the amount of time necessary for recovery. Although many variables contribute to post-thaw hemostasis, the role of platelets are undoubtedly important.

This report describes an in vitro system which can be used to study platelet-blood vessel wall interactions following a freeze-thaw insult. In addition, it provides further insight concerning areas of platelet binding following disruption of the non-thrombogenic endothelial lining.

MATERIALS AND METHODS:

Aorta Source

Thoracic aortas were obtained from freshly slaughtered calves. Each aorta was rinsed at the slaughterhouse to remove residual blood and transported on ice. Three inch aorta segments were excised, intercostal arteries ligated and the ends cannulated with siliconized glass cannulas.

Blood Collection and Plasma Processing

Whole blood was collected from unanestized calves via the jugular vein into plastic centrifuge bottles, containing 25 ml of anticoagulant (acid citrate dextrose, NIH formula A). Platelet rich plasma (PRP) was prepared by centrifugation of the blood bottles at 1500 rpm for 12-14 min at 22°C, while platelet poor plasma (PPP) was collected after centrifugation of PRP at 3300 rpm for 30 min. PPP was used as a blank control for PRP aggregometry and as a dilutent for adjustment of PRP platelet counts.

Gel filtered platelets

Gel filtered platelets (GFP) were used to reduce red blood cell and plasma protein contamination. GFP were prepared as follows (18,29). A plastic column was packed with a slurry of Sepharose 2B gel (Pharmacia Fine Chemicals, Piscataway, NJ), in 0.9% saline containing 0.01% merthiolate. The Sepharose was previously vacuum washed with acetone (3x) followed by saline rinses (5x) to remove impurities (18). Prior to filtration, the column was conditioned by the following elutions: saline (400 ml), Tyrodes with 1% dextrose (200 ml) and complete Tyrodes with 1% dextrose and 3% albumin (200 ml). Complete Tyrodes solution consisted of the following: Na Cl (129 mM), $Na_3C_6H_5O_7$ (10.9 mM), $NaHCO_3$ (8.9 mM),dextrose (1mg/ml), tris base (10 mM), KCl (1.8 mM), KH_2PO_4 (0.81 mM), Mg Cl_2 (0.84 mM), Ca Cl_2 (2.4mM) and bovine albumin (3 mg/ml), (pH 7.3).

After conditioning, PRP (40 ml) was carefully layered on the column and eluted using the complete Tyrodes buffer (40 mls). GFP were stored in 50 ml plastic centrifuge tubes at 22°C until used for aggregometry and subsequent perfusion.

Platelet Processing

Both suspensions of PRP and GFP were counted by hemacytometer and adjusted to a final count of 250,000/mm^3 using PPP or complete Tryodes, respectively. These final suspensions were used for both aggregometry and perfusion.

Aggregometry

Before platelet preparations could be used for perfusion, it was necessary to establish their viability and aggregation potential. This was tested using a Payton aggregometer model 600 (Payton Assoc., Buffalo, NY), and a modification of a previously published procedure (6). Aggregation curves were recorded on an Omni Scribe chart recorder model B5216-2 (Houston Instru., Austin, TX), (P-71). All testing employed a stirring rate of 900 rpm and a temperature of 37°C. Platelet suspensions were tested for their ability to aggregate in response to adenosine diphosphate (ADP) (1 mg/ml, Sigma Chem. Co., St. Louis, MO) and collagen (2.6 mg/ml), (Bio/Data Corp., Horsham, PA). Aggregating agents were added to the cuvettes at a ratio of 1:10 yielding final concentrations of 0.1 mg/ml ADP and 0.26 mg/ml of collagen. Maximum aggregation associated with ADP or collagen was measured over a 10 or 30 min period respectively, and expressed as maximum percent (%) aggregation of the zero baseline value.

Freeze-Thaw Procedure

Both control and experimental aorta segments were filled with complete Tyrodes. Control aortas were maintained submerged in a 37°C water bath during

the course of the experiment. The experimental aortas were first placed in protective plastic bags and submerged in a 95% ethanol refrigerated bath (Neslab, Portsmouth, NH). Bath temperature was lowered at a rate of approximately $1^{o}C$/min. The internal temperature of the aorta lumen was monitored with a thermocouple which had been inserted through one of the intercostal arteries before ligaton. Temperature curves were recorded on a Speedomax chart recorder (Leeds and Northrup, North Wales, PA). When the internal aorta temperatuare reached -15 or $-20^{o}C$, the aorta was immediately thawed in a $37^{o}C$ water bath at a rate of approximately $8^{o}C$/min.

Aorta Perfusion System

A system for perfusing a platelet suspension through the aorta was set up as illustrated in Figure 1. Both control ($37^{o}C$) and experimental (-15 and $-20^{o}C$) aorta segments were perfused under the following conditions: $37^{o}C$, 30 min duration, 120 mmHg and a flow rate of 5 ml/min. After perfusion each aorta was flushed with Ringers for 15 min to remove unattached platelets and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate -0.11M sucrose buffer for 45 min. Flushing and fixation were performed at the same flow rate, temperature and physiological pressure mentioned previously. Fixation was continued overnight (12 hrs) at $22^{o}C$ and 120 mmHg pressure but without flow.

SEM

Following overnight fixation, small segments, from all regions of the aorta, were excised and rinsed in fixation buffer (3x, 15 min ea, $22^{o}C$, pH 7.3). This was followed by postfixation in buffered 1% osium tetroxide (OsO_4) for 1 hr at $4^{o}C$ and buffer rinses (3x, 15 min ea, $22^{o}C$). A graded series of ethanol (ETOH) (70%, 95%, 100%, 100%) was then used to dehydrate the specimens for 10 min ea at $22^{o}C$, followed by critical point drying in liquid CO_2 (Samdri PVT-3, Tousimis Res. Corp., Rockville, MD). Dried aorta segments were mounted on aluminum

stubs, coated with gold/pladium (Technics Hummer 5, Alexandria, VA) and examined in an AMRay 1000 A SEM (Amer. Metals Res; Bedford, MA).

Platelet suspensions were prepared for SEM by prefixing equal volume aliquots of PRP or GFP with 0.2% buffered glutaraldehyde for 30 min at 22°C with aggitation. A 10 ml aliquot was removed for TEM processing while the remaining 10 ml was added to 10 ml of 6% glutaraldehyde (3% final concentration) for final fixation at 22°C for 24 hrs. Following centrifugtion at 3100 rpm for 10 min, the pellet was washed with deionized water (3x, 10 min ea) and dehydrated in a graded series of ETOH. Final resuspension was in 1.0 ml of 100% ETOH prior to placing on glass or plastic coverslips for air drying (22). Finally, platelet preparations were mounted on stubs, sputter coated and viewed with the SEM.

TEM

TEM samples were treated identically to SEM ones through the ETOH dehydration step. After dehydration, small (2 mm) aorta pieces were excised from the ends and middle of each aorta, and placed in propylene oxide (PO) (2x, 30 min ea, 22°C). This was followed by infiltration with PO and Epon-Araldite (EA) embedding resin at ratios of (1:1) and (1:3), respectively, for 1 hr ea. The EA formula used was as previously published (30). Final infiltration occurred in straight EA for 1 hr followed by polymerization for 3 days at 60°C.

Platelet (PRP or GFP) aliquots (10 ml) removed after prefixation for SEM were centrifuged (3100 rpm, 10 min) and the pellet fixed with 3% buffered glutaraldehyde for 2 hrs. The pellet was gently removed with an applicator stick, cut into 1 mm blocks, and washed (2x, 15 min ea) in the same buffer. This was followed by postfixation, buffer rinses and dehydration as described for SEM processing of aorta segments. Platelet blocks were then transferred to PO (2x, 10 min ea) and PO:EA mixtures as described. Final infiltration in EA (1 hr)

proceeded placement in Beem capsules and polymerization for 3 days at 60°C. Ultrathin sections were cut using diamond knives, stained with 5% uranyl acetate (30 min) and 2% lead citrate (6 min), and viewed in a JEOL, TEM, Model 100B (JEOL, Medford, MA).

RESULTS

Aggregometry and Platelet Morphology

The response of PRP and GFP to collagen and ADP induced aggregation are illustrated in Table I. Both PRP and GFP responded quickly to ADP induced aggregation, usually reaching maximal aggregation within 10 min, whereas maximal aggregation with collagen did not occur for 20 to 30 min.

Figures 1 A-D illustrates the morphological appearance of both PRP and GFP as demonstrated by SEM and TEM examination. Platelets from PRP samples were disc shaped (Fig. 1A), with intact dense granules and the presence of plasma proteins (Figure 1B, arrow). Following gel filtration, the platelet morphology was largely disc shaped but there was an increase in the number of sickle shaped platelets (Fig. 1C, arrows). TEM examination revealed an internal morphology identical to the PRP sample with the absence of plasma proteins from the surrounding media (Fig. 1D).

SEM and TEM Examination

The intimal surface of a control (37^{o}C) aorta perfused in the in vitro system (Fig. 2) is illustrated in Fig 3A. The individual endothelial cells were clearly visible, forming a contiguous lining over the aorta surface. In isolated regions where mechanical damage had occurred prior to perfusion, platelets (GFP) could be seen adhering to exposed regions of the subendothelium. (Fig. 3B, arrows). Note that no platelets were attached to the undamaged endothelial cells on the intimal surface.

Following a freeze-thaw insult at either -15 or -20^{o}C, profound changes occurred on the endothelial surface of the aortas (Fig. 4). In all cases, a significant proportion of the endothelial cells were totally removed from aorta surface while in other areas small patches of damaged endothelium remained. GFP could be seen adhering to the exposed basal lamina (Fig. 4, arrows).

Figure 5 (-15°C, GFP) illustrates the numerous platelets which could be seen attached to large areas of exposed subendothelium. Regions of damaged endothelium loosely attached to the internal elastic lamina (IEL) were also found (right side Fig. 5). Note that except for two isolated examples (Fig. 5 insert) platelets were not seen in contact solely with damaged endothelium, but were usually located between endothelial gaps, where subendothelium had been exposed.

A closer examination of several freeze-thaw damaged endothelial cells can be seen in Figs. 6A and B (Fig. 6A, -15°C and GFP perfusion; Fig. 6B, -20°C and PRP perfusion). In both examples, the remaining endothelial cells had damaged plasma membranes with pits and holes over the entire surface (compare to controls in Fig. 3A). Cell border regions were also characterized by large gaps between adjacent cells. Again no platelets (PRP or GFP) were noted in sole contact with damaged endothelium. Platelets adhering to the IEL did not overlap, remained disc shaped, did not undergo aggregation or differ morphologically based on platelet source (e.g. PRP versus GFP). A lower magnification SEM photomicrograph further demonstrated the affinity of GFP for exposed subendothelium as opposed to the freeze-thaw damaged (-15°C) endothelium remaining on the aorta surface (Fig. 7).

Similar findings were noted using TEM. In areas devoid of endothelial cells, after aorta damage (-15°C), attached platelets, still containing their dense granules, could be seen in contact with amorphous material of the basal lamina or LEL (Fig. 8A). Note the presence of collagen fibrils (arrows) beneath successive layers of the IEL but not in contact with attached platelets. A platelet in contact with microfibrils (arrows) of the IEL can be seen in Fig. 8B. Also note that platelets had not undergone degranulation and were not in contact with collagen components of the vessel wall.

An isolated example of a platelet in contact with an endothelial cell is shown in Fig 9. Note the presence of an amorphous material between the platelet and the border region of the endothelial cell (Fig. 9, arrow). No collagen or microfibrils appear to mediate this attachment.

DISCUSSION

The present study was primarily undertaken to develop an in vitro system for the study of freeze-thaw insult to blood vessels and to characterize platelet interactions in such a system after freeze-thaw injury. The in vitro perfusion system illustrated in Fig. 2 proved to be very satisfactory for such a study. It allowed control of temperature, pressure and flow rate parameters. Perfusion under physiological pressure was particularly important since it minimized endothelial artifacts created when blood vessels were not perfused and fixed under physiological conditions (10,12,14).

In the study of platelet-blood vessel wall interactions the use of viable platelet suspensions was of extreme importance. We defined viable platelets as those that demonstrated at least 50% aggregration in response to ADP and collagen. Platelet suspensions which did not meet these criteria were not used for perfusion. Typical aggregation responses shown in Table I demonstrated that both PRP and GFP retained their ability to respond to known aggregating agents following processing.

The effect of plasma proteins on platelet-endothelial interaction was examined by gel filteration of PRP which removed non absorbed plasma protein (29). The SEM and TEM results indicated that the presence (PRP) or absence (GFP) of plasma proteins played no determining factor in platelet-endothelial adhesion or platelet-subendothelial interaction. Platelets from both GFP and PRP suspensions attached to the subendothelium, did not soley interact with damaged endothelium nor undergo degranulation. It should be noted that while gel filtration removes plasma proteins, some fibrinogen remains absorbed on the platelet surface which accounts for their ability to undergo aggregation responses (Dr. Jack, Lindon, personal communication).

In addition, subjective evaluation of SEM micrographs revealed no real differences between aorta segments frozen to -15 or -20°C. At both temperatures severe damage resulted to the intimal surface resulted in with both removal of endothelium and damage to the remaining cells.

The major finding of this study was the lack of any generalized interaction of viable platelets with endothelial cells injured by a freeze-thaw insult. In only rare cases (Figs. 5B, A) were platelets found in sole contact with a damaged endothelial cell as have been described by some investigators using other forms of blood vessel insult (2,15,19,25). Therefore, our results are in general agreement with those who report no generalized platelet-endothelial cell interactions following ischemia (34), vessel ligation (31,32), enzyme treatment (9), and heat and hypotonic treatment of blood vessels (35). While platelets readily adhered to the subendothelium, direct contact with endothelium was almost exclusively limited to regions between adjacent damaged cells. (Fig. 6A, arrow). An example of the rare exception was illustrated in Fig. 9. In this instance, platelet-endothelial cell contact appears mediated by an amorphous material (Fig. 9 arrows) located between the basal surface of the platelet and the apical surface of the damaged endothelial cell. The exact nature of this material is unknown, but does not appear to be microfibril or collagen in nature.

Because platelets did readily adhere to the subendothelium, we examined the subendothelial components found in contact with attached platelets. In large musclular arteries, such as the aorta, the subendothelium immediately underlying the endothelium consists of a thin basal lamina overlying a thick IEL composed mainly of amorphous elastin and microfibrils (24). The TEM micrographs demonstrated that platelets attached to the subendothelium were either in contact with a thin basal lamina (Fig. 8A) or microfibrils of the IEL. These findings in the bovine are in general agreement with other investigators who

have described platelet contact with human glomenular basal lamina (16,17), or subendothelial microfibrils of rabbit aorta (4,5).

No evidence was found to indicate that collagen was responsible for mediating platelet adhesion to the subendothelium. Collagen fibers, easily recognizable in cross or longitudinal section, were found in proximity to the IEL but not in contact with platelets. (Fig. 8A, 8B). This may account for the observed phenomenon that while platelets readily adhered to the subendothelium they did not undergo degranulation or aggregation.

In summary, this in vitro model was determined to be suitable for the study of platelet-vessel wall interactions following freeze-thaw insult. Preliminary findings from such a model indicated that as reported by others, platelets have an affinity only for the subendothelium and not endothelial cells that appear severely damaged by freezing and thawing.

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DISCLAIMER:

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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TABLE I

Aggregating Agent (final concentration)	Maximum Platelet Aggregation (%)* PRP	GFP
ADP (0.1 mg/mml)	64%	71%
Collagen (0.26 mg/ml)	63%	50%

* = Data equal means of nine different experiments

FIGURES

Figure 1A - SEM micrograph of platelets from PRP. x 5,410.

Figure 1B - TEM micrograph of platelets from PRP. Note plasma proteins (arrows). x 12,300.

Figure 1C - SEM micrograph of gel filtered platelets. x 5,860.

Figure 1D - TEM micrograph of gel filtered platelets. Note absence of plasma proteins. x 12,600.

Figure 2 - In vitro perfusion apparatus used to perfuse bovine aorta segments with platelet suspensions.

Figure 3A - Bovine aorta wall maintained at 37°C and perfused with GFP. Note intact endothelial cells and lack of adhering platelets. x 2,500.

Figure 3B - Bovine aorta wall perfused with GFP at 37°C. Note region of mechanical trauma with platelets adhering to the subendothelium, but not to intact endothelial cells. x 2,080.

Figure 4 - Aorta freeze-thawed (-20°C) and perfused with GFP. Note many platelets attached to subendothelium where endothelial cells have been removed. x 1,040.

Figure 5 - SEM micrograph of aorta wall freeze-thawed (-15°C) and perfused with GFP. Numerous platelets are seen sticking to the subendothelium. Note patch of damaged endothelium (right side) and two platelets attached to an endothelial cell surface (arrows). x 2,220.

Figure 5 Insert - Higher magnification insert of a portion of Figure 5 demonstrating two platelets (GFP) in sole contact with damaged endothelial cell. x 8,430.

Figure 6A - SEM micrograph showing close up of two freeze-thaw (-15°C) damaged endothelium. Note pits and holes in the plasma membrane. Platelets (GFP) are in contact with the IEl but not the endothelial cells. x 5,305.

Figure 6B - A similar micrograph to Fig. 7A but sample was frozen at (-20°C) and perfused with PRP. Note that platelet appearance does not differ from GFP. x 5,340

Figure 7 - SEM micrograph of GFP adhesion to the exposed subendothelium of a freeze-thawed (-15°C) aorta. Note platelets do not interact with small patch of remaining endothelium, but form a total monolayer over the IEL. x 2,485.

Figure 8A - TEM micrograph of platelets (GFP) attached to amorphous constituents of the basal lamina following freze-thaw (-15°C). x 31,800.

Figure 8B - TEM micrograph of platelets (GFP) attached to microfibrils of the IEL following freze-thaw (-15°C). x 22,500.

Figure 9 - TEM micrograph of platelet (PRP) attached to the border region of a damaged (-15°C) endothelial cell. Note presence of an amorphous material (arrows) mediating cell to cell contact and absence of collagen (C) involvement. x 22,100.

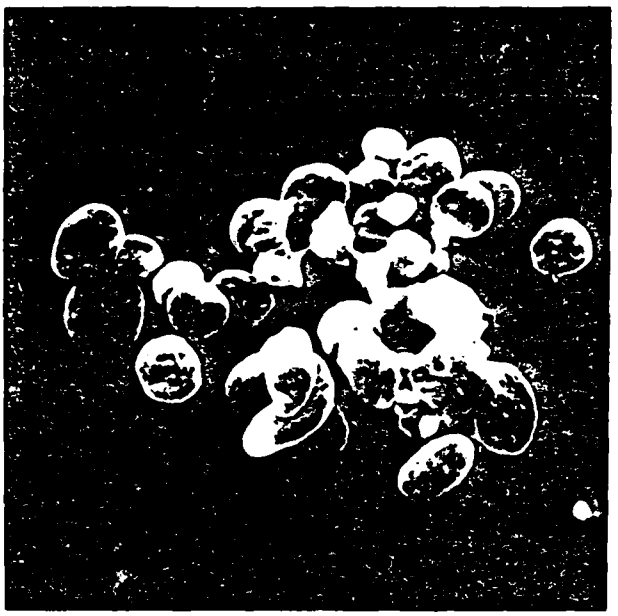


Fig. 1A

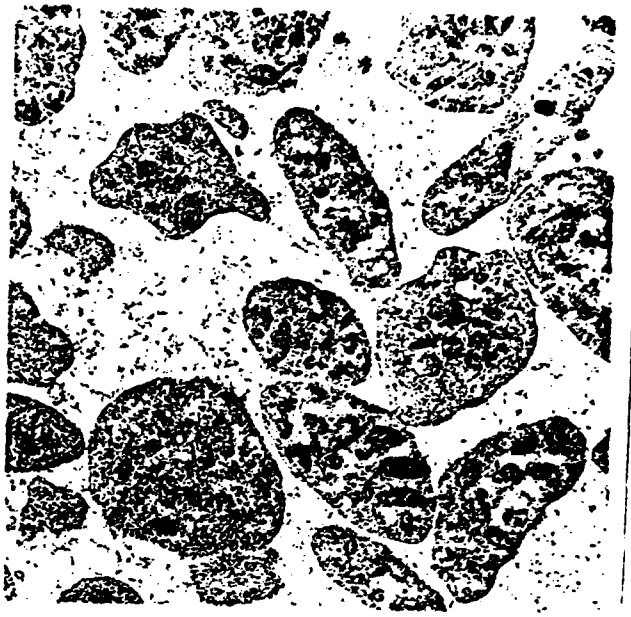


Fig. 1B

Fig. 1C

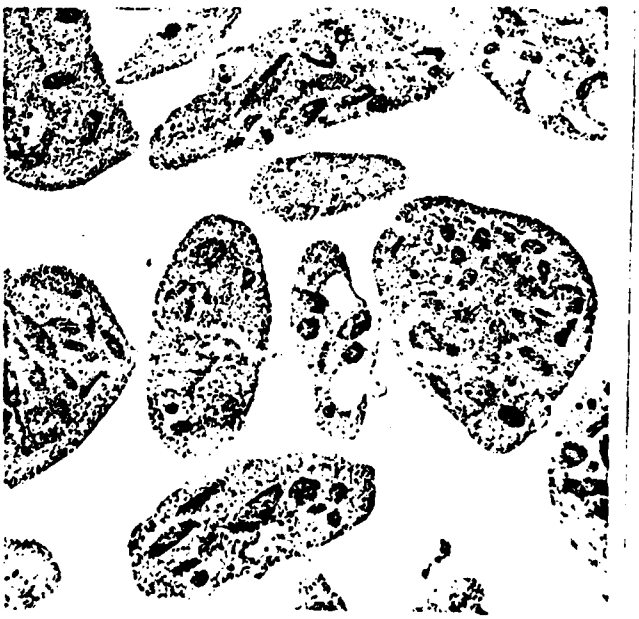


Fig 1D

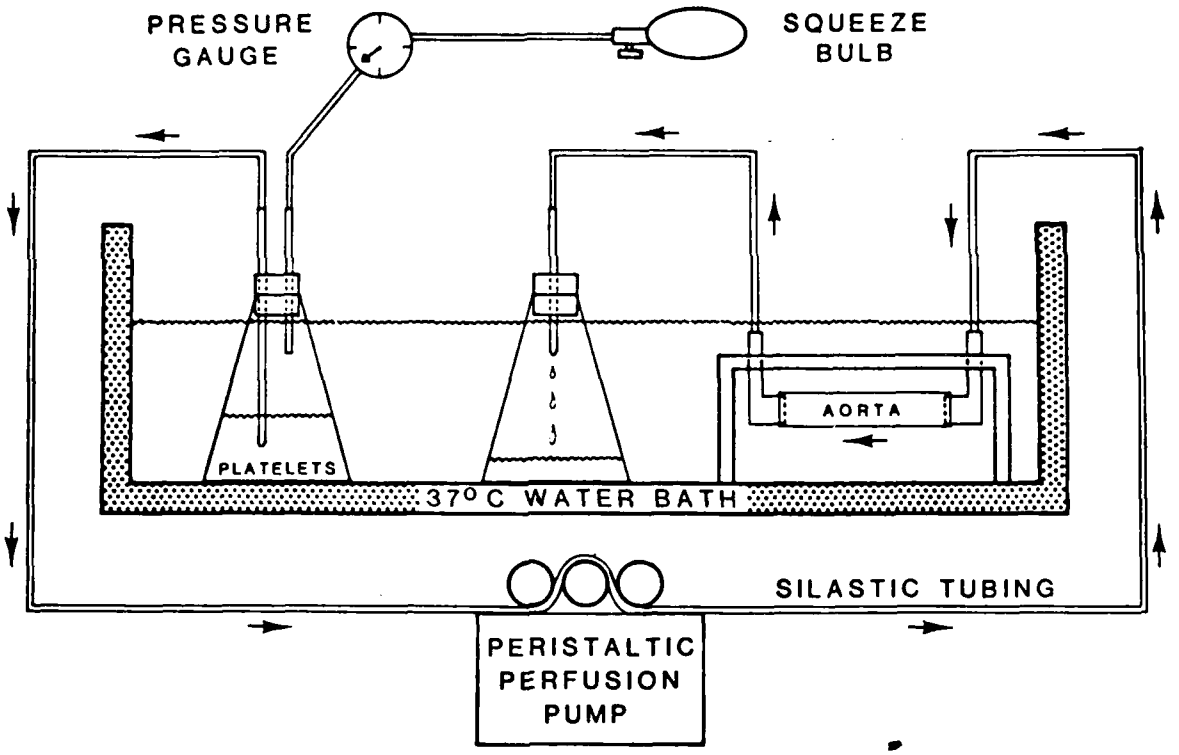


Fig. 2

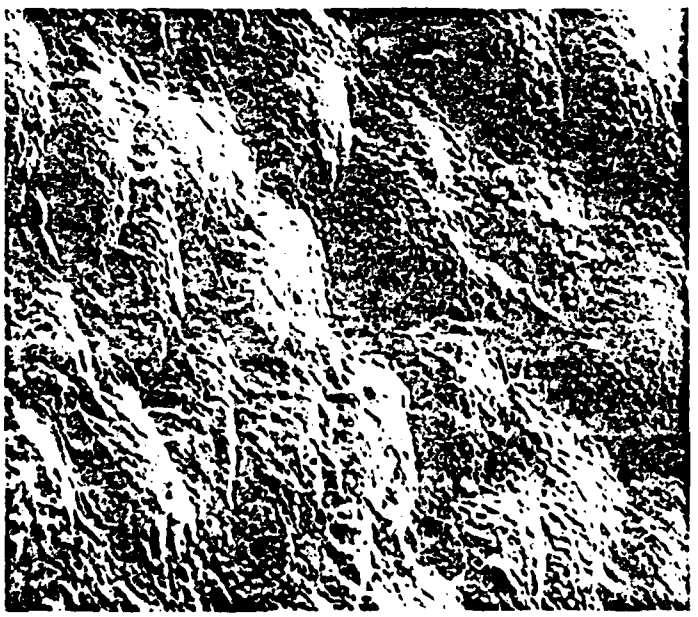
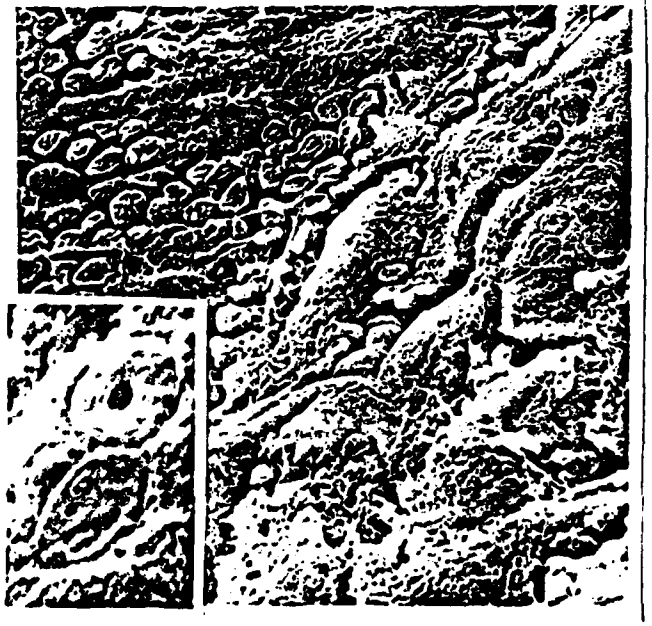


Fig. 3A

Fig. 3B

Fig. 4



Insert

Fig. 5

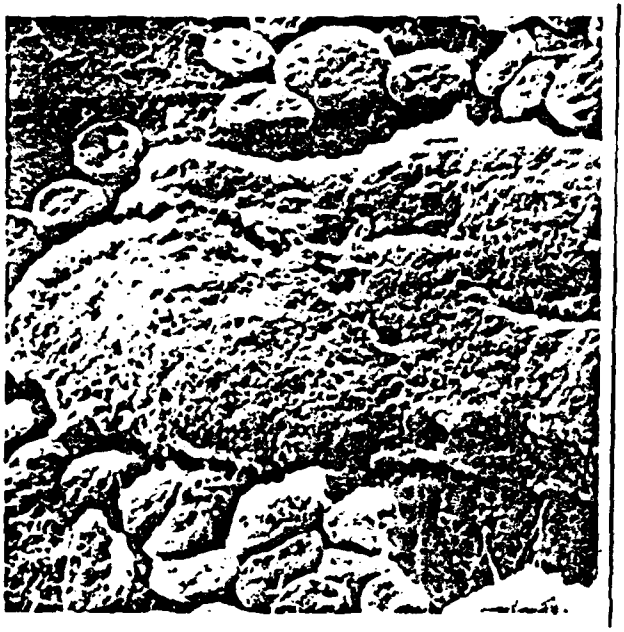


Fig. 6A

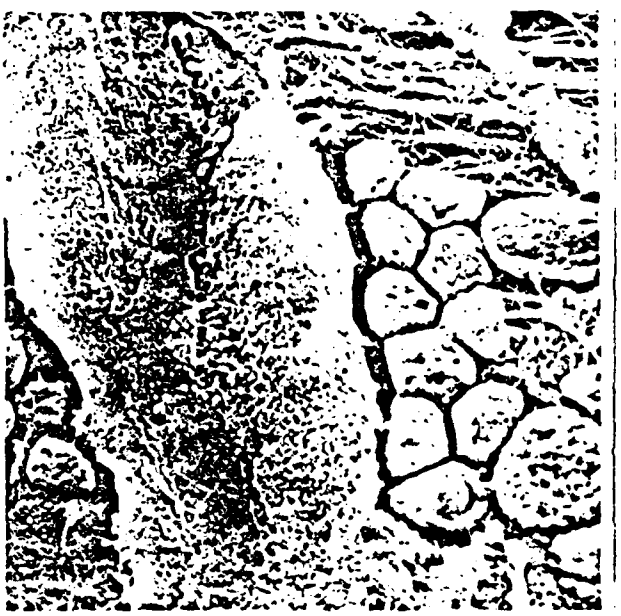


Fig. 6B

Fig. 7

Fig. 8A

Fig. 8B

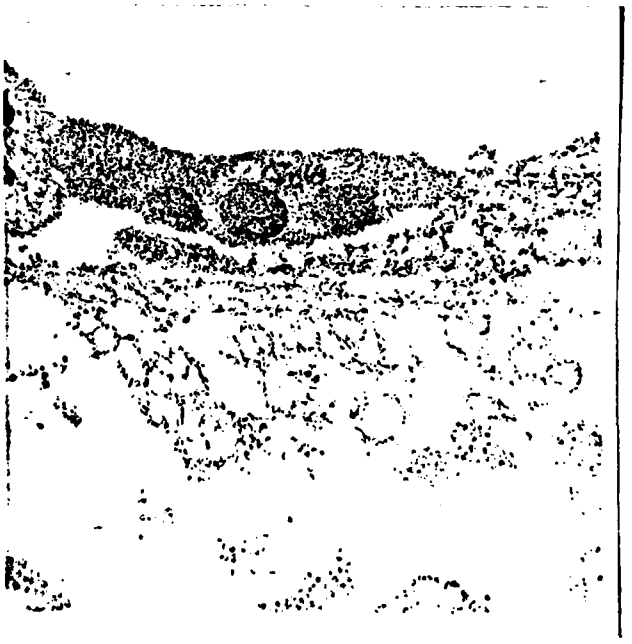


Fig. 9

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